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Genomic comparison of the complete coding and intergenic regions of the VG/GA Newcastle disease virus and its respirotropic clone 5

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Abstract The complete genome of the Villegas-Glisson/University of Georgia (VG/GA) strain of Newcastle disease virus (NDV) and that of a plaque purified clone (clone 5) exhibiting a respiratory phenotype were sequenced and analyzed. The VG/GA strain, isolated from the intestine of healthy turkeys, replicates in the respiratory and intestinal tract of chickens. It is used worldwide as a vaccine strain and its tissue tropism is extremely important for protection against velogenic viscerotropic NDV which targets both intestinal and respiratory epithelia, inducing severe gross and microscopic damage. The clone 5, a plaque purified clone from the VG/GA strain, cannot be recovered from the intestine of infected birds, suggesting a respirotropic nature. A modified primer sequence-independent amplification method was used to sequence the complete coding regions of both viruses and to assess phylogenetic relationships and genomic basis for phenotype differences. The phylogenetic analysis grouped the VG/GA strain and the clone 5 within class II, genotype II viruses and showed that they are greater than 99.9% identical with only 5 nucleotides differences. Both are closely related to classic vaccine strains, such as LaSota and B1. Only 3 amino acid differences at the fusion protein differentiated the VG/GA strain from the clone 5. These differences may explain the differential phenotype observed in the VG/GA strain and are discussed.

Keywords Newcastle disease virus · VG/GA strain · Clone 5 strain · Full genome · Sequencing · Analysis

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Introduction

Newcastle disease virus (NDV) is one of the most important infectious agents in poultry, affecting a wide variety of birds and causing important economic losses [1]. The virus belongs to the family *Paramyxoviridae*, subfamily *Paramyxovirinae,* in the genus *Avulavirus* [2], and encompasses a diverse group of single-stranded, negative-sense, nonsegmented RNA viruses of approximately 15.2 kb. From the NDV genome (3′–5′ terminus), six structural proteins are produced: nucleocapsid (N gene), phosphoprotein (P gene), matrix (M gene), fusion (F gene), hemagglutinin-neuraminidase (HN gene), and the RNA-dependent RNA polymerase (L gene) [3]. Two nonstructural V and W proteins generated by RNA editing from the P gene are also recognized [4–6]. The F protein induces fusion of the viral and host cell membranes, and the HN protein possesses both receptor recognition of sialic acid at the termini of host glycoconjugates and neuraminidase activity to hydrolyze sialic acid from progeny virion particles to prevent viral self-aggregation [3, 7, 8]. In addition to these activities, the HN protein has been shown to promote fusion through its interaction with the F protein, thereby allowing the entry of viral RNA [9, 10].

Newcastle disease (ND) vaccines to control detrimental effects of sub-clinical forms of the disease and extremely expensive outbreaks are commonly used. The poultry industry relies on vaccination programs using live and inactivated lentogenic strains, as well as biosecurity measures, to diminish the economic impact of the disease [7, 11–13].

The Villegas-Glisson/University of Georgia (VG/GA) strain of NDV was isolated in 1987 from the intestinal tract of turkeys showing no signs of respiratory disease, and it is commonly used as a vaccine strain worldwide. Initial investigations with the VG/GA strain stated that the virus is a naturally attenuated lentogenic strain that produces no detectable respiratory reaction in chickens regardless of the vaccination route used, and that it affords respiratory tract protection against challenge with a respiratory-type NDV (LaSota strain) that produces high hemagglutination inhibition titers showing no interference with immunity to infectious bronchitis [14, 15]. The VG/GA virus replicates both in the respiratory and intestinal tract, with preference for the intestine [15, 16]. After vaccination with the VG/GA strain, solid immunity to virulent NDV is conferred to inoculated chickens with or without the presence of maternal antibodies as evidenced by resistance to virulent NDV challenge [15, 17]. Additionally, the vaccine-induced respiratory reactions are decreased or avoided when using the VG/GA strain, probably by diminishing the level of replication in the respiratory epithelia of young chickens [18].

Several plaque purified clones of the VG/GA strain were developed and tested for virus isolation in the respiratory and intestinal tract of chickens. The isolate designated as clone 5 could not be recovered from the intestinal tract and hence was selected as an alternative vaccine candidate, currently used as a vaccine strain in the US. The VG/GA strain tissue tropism has been evaluated by immunohistochemistry [16] and reverse transcriptase-polymerase chain reaction (RT-PCR) [15], confirming that it can be detected both in the respiratory and intestinal tract of chickens, while clone 5 similar to other classic vaccine strains such as LaSota and B1 is detected mostly in the respiratory tract [19].

There is evidence of extensive variability among single stranded negative sense RNA viruses that is represented by both synonymous and nonsynonymous mutations within the same virus population [3, 20]. The fact that the closely related VG/GA and clone 5 exhibit different tissue tropism may allow the identification of specific amino acid changes associated with the phenotype of the viruses. The aim of this work was to sequence and compare the complete coding regions of the VG/GA and clone 5 viruses in an attempt to and to assess the genomic basis of their different tissue tropism.

Materials and methods

Viruses

The VG/GA/turkey/1987 strain used in this trial was the third embryo passage of the original isolate. Among several VG/GA strain plaque purified clones, the one designated as clone 5 was selected because it could not be recovered from the intestine of experimentally inoculated specific pathogen free (SPF) chickens. The virus replicates in the upper respiratory tract and could be re-isolated from tracheal swabs. Both viruses were propagated by inoculation into 9-day old chicken embryos. Titers of 10^8 embryo infective dose 50/ml (EID_{50}/ml) were obtained. The allantoic fluid (AF) stock virus was kept at −80°C until used.

RNA extraction and genome amplification

RNA was extracted from VG/GA and clone 5 allantoic fluids using Trizol LS (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After RNA extraction, cDNAs were generated and amplified using a modification of a novel random priming viral genome sequencing method described by Djikeng and coworkers [21]. A denatured set of primers designated as FR: **FR26RV-N** 5′-GCCGGAGCT CTG CAGATATCNNNN NN-3′ and **FR20RV** 5′-GCCGGAGCTCTGCAGATATC-3′ were used. The modification consisted in the addition of a pair of primers (beg2/end-r): **beg2** CGCGTCGACTAGT ACGGGTAGA and **end-r** GTACCCGGGGATCCTTTTT TCTA to increase coverage at the 5′ and 3′ends. The excised segments were eluted using the QIAquick Gel extraction kit (QIAGEN) following the manufacturer's instructions. TOPO TA cloning was applied to these segments and individual colonies allowed to grow in 96-well plates, after which a standard plasmid purification protocol was followed. Forward and reverse sequencing was applied to a total of 384 sequences per virus.

Sequence data, nucleotide sequencing, and alignment analysis

All nucleotide sequencing reactions were performed with fluorescent dideoxynucleotide terminators in an automated ABI sequencer (ABI 3730 automated sequencer; Applied Biosystems Inc., Foster City, CA). The average nucleotide coverage for sequencing was 14.7 for VG/GA and 14.5 for clone 5. Nucleotide sequence assembly and editing were conducted with the CodonCode Aligner (Codon Code Corporation (Deham MA) sequence assembly software using Phred-Phrap algorithms. Comparison sequences were retrieved from GenBank public databases and used to generate alignments. Accession numbers for the NDV

sequences used for comparison and alignment are summarized in Table 1. Alignments of complete genomes were performed using BioEdit v. 5.0.9 (Department of Microbiology, North Carolina State University, Raleigh) with the ClustalW program followed by manual editing. Phylogenetic tree construction was done with Phyml V2.4.4 (for bootstrap analysis) under the GTR model of nucleotide substitution with estimated proportions of invariable sites, ML base frequencies estimates, four substitution rate categories, and an affixed gamma distribution parameter. Coding regions were identified by translating the corresponding open reading frame with Bioedit and proteins alignments were performed with the ClustalW program. Amino acid distances among poultry vaccine virus strains

Table 1 Viruses and accession numbers used for comparison and alignments

Abbreviation[a]	Species	Location	Identification	GenBank accession
98AUCKN-1	Chicken	Australia	98–1154	AY935491
99AUCKN-1	Chicken	Australia	99-0868-1	AY935495
99AUCKN-2	Chicken	Australia	99-0868-2	AY935496
99AUCKN-3	Chicken	Australia	99-1997 (PR-32)	AY935497
98AUCKN-2	Chicken	Australia	98-1249	AY935492
98AUCKN-3	Chicken	Australia	98-1252	AY935493
99AUCKN-3	Chicken	Australia	99-0655	AY935494
99AUCKN-4	Chicken	Australia	99-1435	AY935498
01AUCKN	Chicken	Australia	01-1108	AY935489
02AUCKN	Chicken	Australia	02-1334	AY935490
06AUCKN1	Chicken	Australia	I-2 progenitor	AY935500
06AUCKN2	Chicken	Australia	I-2	AY935499
67NICKN	Chicken	N.Ireland	Ulster/67	AY562991
92CNCKN	Chicken	China	HB92-V4	AY225110
07KOCKN	Chicken	Korea	KBNP-4152	EU140955
05CNCKN	Chicken	China	AQI-ND026	DQ060053
48USCKN-1	Chicken	USA	LaSota	AF077761
48USCKN-2	Chicken	USA	LaSota	AY845400
99USCKN	Chicken	USA	LaSota-Clone 30	Y18898
47USCKN-1	Chicken	USA	B1-Hitchner	AF309418
47USCKN-2	Chicken	USA	B1-Hitchner	NC002617
47USCKN-3	Chicken	USA	B1-Takaaki	AF375823
07USTKY	Turkey	USA	VG/GA-Clone V	EU289029
89USTKY	Turkey	USA	VG/GA	EU289028
45INCKN	Chicken	India	Mukteswar	EF201805
33ENCKN	Chicken	England	Herts/33	AY741404
93USANH	Anhinga	USA	anhinga/FL	AY562986
71USMIX	Mixed	USA	Largo/71	AY562990
02USGWL	Gamefowl	USA	Gamefowl/Cal/02	AY562987
00ITDVE	Dove	Italy	Dove/italy/00	AY562989
82ITPGN	Pigeon	Italy	IT-227/82	AJ880277
72USCKN	Chicken	US	Fontana	AY562988
90IDCOC	Cockatoo	Indonesia	Cockatoo/Indonesia	AY562985
06CNGSE	Goose	China	NA1	DQ659677
03CNCKN1	Chicken	China	Guangxi-9	DQ485230
02CNCKN	Chicken	China	Guangxi-7	DQ485229
07CNGSE	Goose	China	Zj1	AF431744
03CNCKN2	Chicken	China	Guangxi-11	DQ485231
06CNCKN	Chicken	China	GM	DQ486859

[a] Abbreviation: year of isolation or submission, country, specie

were determined based on 4,556 amino acids corresponding to the concatenated coding regions of the NC, P, M,F, HN, and L genes, which were aligned and analyzed using the NJ method. Analysis included 1,000 bootstraps replications, amino acid Poisson correction with homogeneous pattern among lineages, and uniform rates among sites. The random approach provided the entire genomic sequences with the exception of the noncoding terminal ends. It provided genomic sequence up to approximately 50 bps from each end of the genomes; the 5′ and 3′ noncoding regions were sequenced from PCR amplified products using the primers F5′-ACCAAACAGAGAATCCGTGAG TTA-3′, R5′-GATCCTTCTCGTTTCTGACTCATC-3′, and F5-′AGCTGAGGGTGATCTCGCTGACAC-3′, R5′ CCAA CAAAGATTTGGTGAATGACG-3′, respectively.

Results and discussion

Random priming and cDNA synthesis of VG/GA and clone 5 genomes produced multiples fragments of size ranging from 200 to 12,000 bases pairs. cDNAs were size fractionated using gel electrophoresis and for the FR set of primers all inserts > 500 bp were excised in two fragments (bands 500–1,000 bp and bands above 1,000); for the beg2/end-r set one fragment above 500 bp was excised (Fig. 1). The excised fragments were eluted and cloned into plasmids and sequenced as described in the material and methods section. Plasmids with NDV inserts were assembled into two single contigs containing the entire coding sequences of VG/GA and clone 5, respectively. Phylogenetic comparisons of the VG/GA and clone 5 genome sequences with NDV full genome sequences available in the GenBank are shown in Fig. 2. The VG/GA and the clone 5 were 99.9% identical with only five nucleotides changes in the entire genome. These strains were more closely related to the LaSota and B1 lentogenic viruses isolated in the U. S during the late 1940s and more distantly related to other vaccine viruses u, such as the HB92 derivative of the Queensland V4 strain and the I2 strain. Since the VG/GA strain was isolated from the intestine of healthy turkeys that usually are exposed to LaSota and B1 viruses, it seems possible that the VG/GA strain shares a common ancestor with them, or that the VG/GA and clone 5 are derivatives from these viruses that circulated in the US during the 1940s and are currently used as vaccine strains. Similar results have been previously reported using partial sequences of the VG/GA genome for the phylogenetic analyses [22–24].

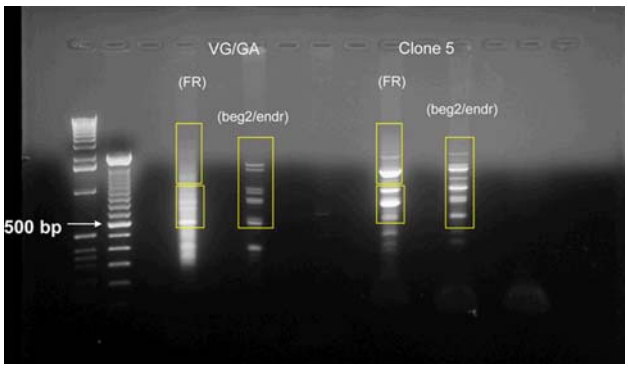

Fig. 1 Size distribution of cDNAs obtained from random RT-PCR. cDNAs were size fractionated using gel electrophoresis and all inserts >500 bp excised in two fragments (bands 500–1,000 bp and bands above 1,000) for the FR set of primers and one fragment above 500 bp for the beg2-end-r set . The excised segments (boxed) were eluted and TOPO TA cloning was applied to these segments; individual colonies were allowed to grow in 96-well plates, after which a standard plasmid purification protocol was followed

Phylogenetic analyses based on the concatenated amino acid sequence of six proteins (N, P, M, F, HN, and L) revealed that the VG/GA and clone 5 viruses grouped within the class II, genotype II and likely correspond antigenically with most of the classic vaccine strains used in the poultry industry. Nevertheless, they differed from the other lentogenic strains with enteric tropism used for poultry vaccination (V4, Ulster, PHY-LMV42 and Queensland V4); these viruses belong to the class II in the genotype I (Fig. 3). The Ulster V4 derivatives, PHY-LMV42, and Queensland V4 strains are more closely related to the Australian viral isolates of genotype I, for which natural drift mutations at the cleavage site responsible for increases in virulence have been reported [25].

The F glycoprotein of NDV is a type I integral membrane protein shown to be involved in virus penetration and cell fusion [26]. For the viruses analyzed, the amino acid differences in the F protein included a Methionine (M) at position 108 in the VG/GA virus substituting a Valine (V) in clone 5 and in all the vaccine strains used for comparison (Fig. 4). The I2, PHY-LMV42, and Ulster 67 (all enterotropic strains) showed an Arginine (R) to a Lysine (K) substitution at position 115 when compared to the VG/GA and clone 5. Other important amino acid changes included substitutions in the VG/GA strain at positions 135 and 136 which belong to the N-terminus of the F1 section of the F protein (fusion peptide). At these positions, the VG/GA virus presented two contiguous nonpolar amino acids [Methionine (M) and Alanine (A)]. Meanwhile, the clone 5, together with most of the paramyxoviruses [27] including lentogenic and velogenic strains of NDV, showed a nonpolar Isoleucine (I) and a polar Threonine (T) at those positions. The fusion peptide is a highly conserved structure among *Paramyxovirus* (up to 90% identity) and is directly involved in fusion promotion [3, 27]. The significance of these changes in NDV tissue tropism is yet to be determined; nevertheless, it is known that amino acid

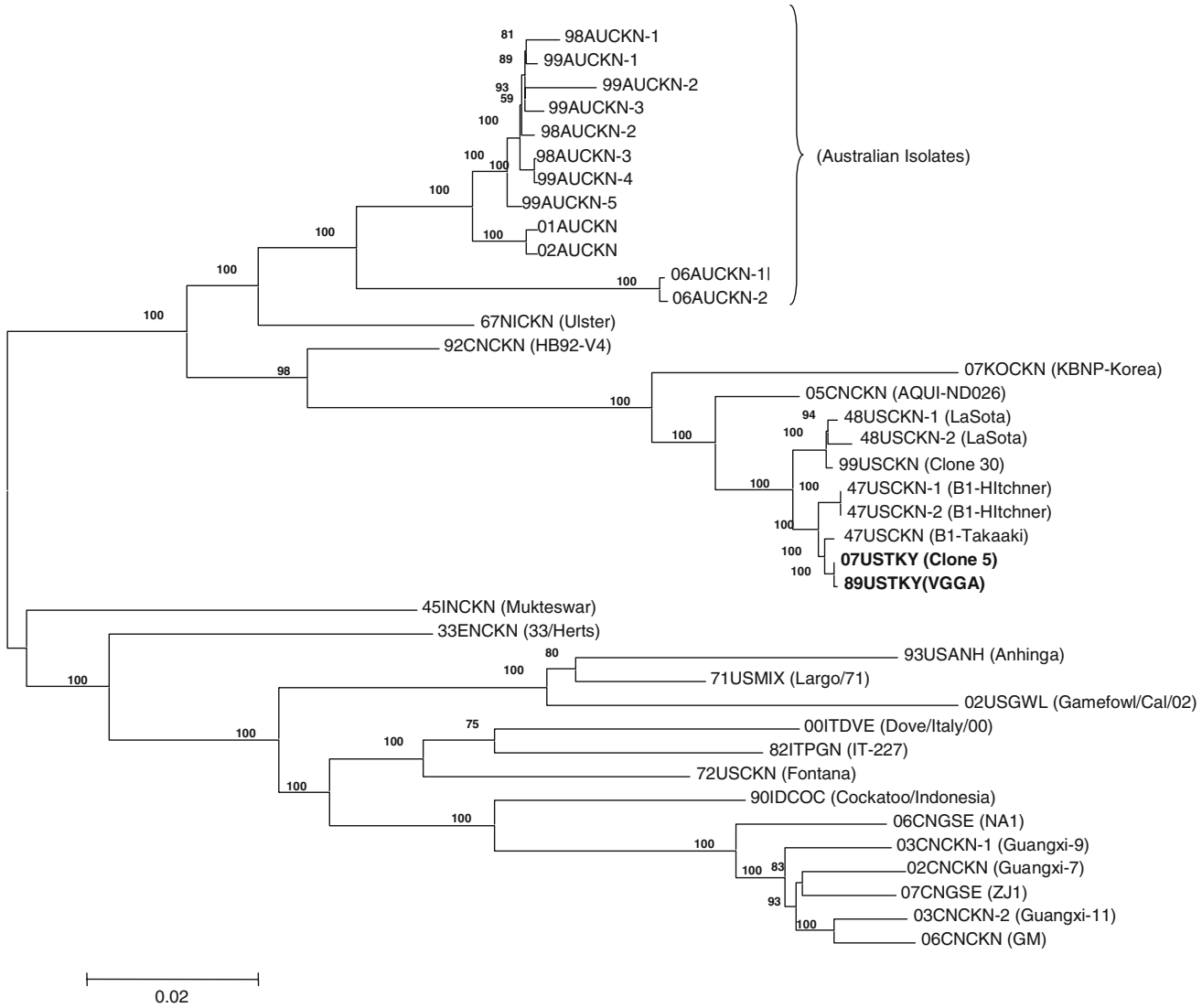

Fig. 2 Complete genome phylogenetic comparison among VG/GA, clone 5, and full genome NDV sequences available from GenBank

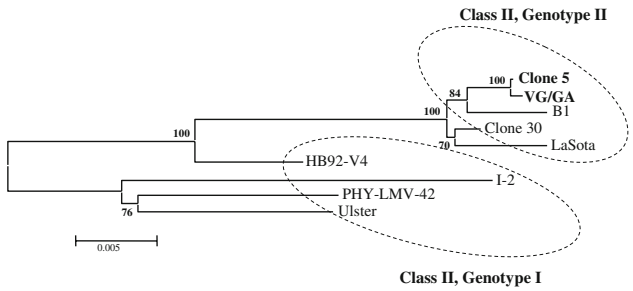

Fig. 3 Phylogenetic analysis of vaccine viruses based on amino acid sequences. 4,556 concatenated amino acids sequences corresponding to the NC, P, M,F, HN, and L proteins were aligned with ClustalW, manually edited, and analyzed using the Neighbour Joining method. Analysis included 1,000 bootstraps replications, using amino acid Poisson correction, homogeneous pattern among lineages, and uniform rates among sites

substitutions at specific locations can have a profound effect on the folding and function of the proteins, and the mutations observed in the F protein of the VG/GA strain are unique and may be associated with its phenotype.

Romer-Oberdofer and coworkers [28] indicated in 2003 that the length of the HN protein may play a role in the ability of the virus to spread and propagate in various organs after inoculation. The 577 amino acids of the VG/GA and clone 5 HN proteins are of the same length of the LaSota HN protein and other virulent and nonvirulent strains, which may indicate that in the VG/GA and clone 5, the length of HN may not be a factor in tissue tropism.

When compared with LaSota and B1 strains, the VG/GA and clone 5 L genes revealed a single nucleotide insertion at position 3,870, compensated by a nucleotide deletion downstream at position 3,958. These changes resulted in a 30-amino acid substitution in the domain V of the L protein

Fig. 4 Amino acid alignment of selected regions of the F, HN and L proteins of NDV vaccine viruses. F protein residues 106–136; HN protein residues 168–205; L protein residues 1,285–1,318. (Virus abbreviations are as indicated in Table 1)

(Fig. 4). This mutation has been documented in velogenic and lentogenic NDV viruses, including most of the enterotropic strains, such as I2, PHY-LMV42, and Ulster 67 [29]. Kusumaningtyas and coworkers [29] reported the existence of two forms of the L protein of NDV after sequence analysis of NDV isolates from different backgrounds. The association of this genotype with virus replication and tissue tropism is yet to be determined for NDV; however, domain V of the L protein has been proposed to play an important role in transcription and thermosensitivity of isolates of vesicular stomatitis virus and Sendai virus [30, 31]. An interesting observation is that the clone 30 strain which is an attenuated form of LaSota strain of NDV has the same amino acid sequence as the VG/GA and the clone 5 strains in this segment of the L protein [32]. Kattenbelt and coworkers [33] discussed the role of the L protein in NDV thermostability indicating that RNA polymerase is required for transcription of mRNAs and full genome-length positive sense RNA and that if the virion is exposed to environmental (intestinal cell) heat, the RNA polymerase may be rendered inactive, and although attachment and entry of that virus particle may still occur following infection of a host, transcription would not occur and virus replication would be terminated. They claimed that it is possible that changes to the structure or configuration of the RNA polymerase allow this protein to retain activity when the virion is subjected to heat.

In summary, the full genome analysis grouped the VG/GA strain and the clone 5 within class II, genotype II viruses and showed that they are closely related to classic vaccine strains, such as LaSota and B1. Differences at the nucleotide and amino acid level were observed between the VG/GA strain and the clone 5 and between these and other vaccine strains. Although it is not possible to correlate specific genomic changes to the restrictions in replication of clone 5, it is remarkable that a small number of changes appear to be sufficient to prevent replication in the intestinal track, and this demonstrated the need for further studies using reverse genetics and nucleotide substitution assays.

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